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13. ABSTRACT (Maximum 200 Words)

Tamoxifen, a SERM (Selective Estrogen Receptor Modulator), is the most commonly used endocrine treatment for all stages of breast cancer. However, progression from tamoxifen sensitivity to tamoxifen resistance occurs in a substantial portion of the tumors. Full antiestrogens, such as ICI 182,780, are currently used as the second line therapy after failure of long-term tamoxifen therapy. To facilitate the design and characterization of more appropriate therapeutic agents for endocrine therapy of breast cancer, it is very important to understand the functional mechanisms that distinguish full antiestrogens from SERMs. It has been shown that estrogen receptor (ER) can recruit corepressors NCoR (nuclear receptor corepressor) and SMRT (silencing mediator of retinoid and thyroid receptors) in the presence of tamoxifen, suggesting a possible role of N-CoR/SMRT in mediating the antagonist activity of tamoxifen. However, it is not clear if apo-ER or ICI 182,780-bound ER can recruit NCoR/SMRT or other corepressors. To investigate the possible involvement of different corepressors in the actions of different antiestrogens and unliganded ER, we have constructed a focused phage display library which contains the "CoRNR box" motif, a binding site important for N-CoR/SMRT to interact with the nuclear receptors. In this report, we have shown that screening of the CoRNR box library with ER treated with no hormone or different antiestrogens led to the isolation of peptides that differentially interact with apo-ER, tamoxifen-bound ER, or ICI 182,780-bound ER. These interactions observed *in vitro* have also been confirmed *in vivo* using a mammalian two-hybrid assay. Using a series of ER mutants, we were able to show that these CoRNR box-containing peptides have different binding characteristics from the peptides that contain the coactivator LXXLL motif. These peptides can be used to probe the conformational changes of ER induced by different antiestrogens and will be valuable for the design of screens for novel ER-antagonists.

14. SUBJECT TERMS
Tamoxifen, ICI 182,780, estrogen receptor, corepressor, NCoR, SMRT, proteomics, phage display

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Introduction

The antiestrogen tamoxifen is the most commonly used endocrine treatment for all stages of breast cancer. Tamoxifen is a SERM (Selective Estrogen Receptor Modulator) which can act as an estrogen or an antiestrogen depending on the tissues in which it operates. Initially, tamoxifen may have profound effects on the survival and proliferation of breast cancer cells, but progressively, resistance emerges through mechanisms that are only partially understood. Some pure antiestrogens lack cross-resistance with tamoxifen, thus makes them good candidates as second-line therapy for patients who develop tamoxifen resistance and also suggests that the mechanisms underlying the actions of SERMs and pure antagonists are very different. This proposal investigates the possible involvement of different corepressor proteins in mediating the actions of different classes of antiestrogens. To probe the changes in the structure of the estrogen receptor (ER) upon binding of different antiestrogens, a focused phage displayed peptide library which expresses peptides containing a "CoRNR box" motif, important for corepressor to bind nuclear receptor, was made. Peptides that differentially interact with ER occupied by different antiestrogens were obtained. These peptides had provided a better understanding of actions of different antiestrogens and the assay which detects the peptide-ER interaction can be used as a sensitive and efficient system to screen for novel antiestrogens.

Body

In the previous two granting periods, we identified CoRNR box pepitdes which interact with ER only in the absence of hormone or in the presence of antiestrogens. Interaction of these CoRNR peptides with ER was also confirmed in cells using mammalian two-hybrid assay. A series of ER mutants were generated to define the regions within ER required for CoRNR box peptide binding. For comparison, LXXLL motif-containing peptides were also included. The LXXLL is a motif found in all p160 coactivators and required for these coactivators to interact with the estrogen receptor. The LXXLL peptides used in this study have been previously shown to interact with ER only in the presence of agonist but not in the presence of SERMs or pure antagonists (1). Mutations at the amino acid residues Lys-362 and Val-376 within ERα decreased its ability to interact with both the CoRNR and LXXLL peptides, suggesting that the binding surfaces for these two classes of peptides are close or overlapping. Mutation at Leu-372, however, totally abolished the interaction between ERα and two of the CoRNR peptides (bT1 and bI2) while having no effect on binding of the LXXLL peptides to the receptor. Thus, although the binding sites on ERα for the CoRNR and LXXLL peptides are closely linked, they can be functionally separated.

The major task in this last granting period is to further dissect the mechanism of ER action using CoRNR peptides and ER-L372R.

- **Examination of the possible involvement of corepressors other than NCoR/SMRT in regulating the transcriptional activity of ER**

There is strong evidence that corepressors NCoR and SMRT can modulate the agonist activity of tamoxifen, but they play little, if any, role in regulating the physiological actions of estradiol (2, 3). In the 2004 annual summary, we had reported that one ERα mutant, ER-L372R, lost the ability to interact with two of the CoRNR box-containing peptides. Binding of ER-L372R to LXXLL motif-containing peptides, however, was not affected. Predictably, we observed that the agonist activity of tamoxifen was enhanced by disruption of the putative corepressor binding surface (defined by the L372R mutation) on ERα. However, one of the most interesting findings of our study was that this mutation also enhanced the agonist efficacy of estradiol. One possible explanation is that this mutation disrupts the interaction of ER with a corepressor (or a competitive repressor) that, unlike NCoR and SMRT, can recognize the agonist-activated structure of ERα. One potential candidate that could function in this manner is REA (repressor of estrogen receptor activity).

REA potentiates the inhibitory activity of antiestrogen-liganded ER and represses the transcriptional activity of the estradiol-liganded ER (4). Neutralization of endogenous REA using antisense REA enhances ER transcriptional activity mediated by estradiol (5). It has been shown that REA can interact with ER in the presence of estradiol or tamoxifen, which dampens the stimulatory response of ER to these two ligands. REA-interacting domain of the ER has been localized between amino acids 304-530. The observation of the enhanced transcriptional activity of ER-L372R led us to hypothesize that REA may interact with ER through Leu-372. To test this hypothesis, HepG2 cells were transfected with an expression vector for the wild-type ER (ER-WT) or ER-L372R and the reporter construct 3xERE-TATA-Luc in the absence or presence of increasing amounts of an expression vector for REA and cells were then treated with estradiol. As shown in Figure 1A, REA can repress 30% of the estradiol-mediated wild-type ER

transcriptional activity. If Leu-372 is important for interacting with REA, mutation at this amino acid should disrupt the REA-ER interaction and REA can no longer inhibit ER activity. However, Figure 1B showed that REA can also suppress 30% of the ER-L372 activity induced by estradiol. It is possible that other corepressors may be involved in mediating ER activity through Leu-372.

- **Increased interaction with known LXXLL motif-containing peptides may partly account for the enhanced transcriptional activity of the ER-L372R mutant**

Another possible explanation for the enhanced transcriptional activity of ER-L372R is that mutation of Leu-372 to an arginine may inadvertently increase the affinity of the receptor for coactivators by making additional contacts with the amino acid residues adjacent to the LXXLL motif. To test this hypothesis, the abilities of wild-type ERα and ER-L372R to interact with different classes of LXXLL-containing peptides were compared in the presence of increasing concentrations of estradiol using a mammalian two-hybrid assay (Figure 2). Three LXXLL peptides D11, D47, and F6 with distinct receptor binding characteristics (1) were used. The GRIP1 NR-box peptide contains the middle three copies of the LXXLL motif found in the coactivator GRIP1. The SRC-1 NR-box peptide contains the middle three copies of the LXXLL motif found in the coactivator SRC-1. As shown in Figure 2, compared with the wild-type ERα, the ER-L372R mutant did not show a higher affinity for any of the LXXLL peptides tested. However, ER-L372R did exhibit a higher capacity for binding to the GRIP1 NR-box peptide at higher concentrations of estradiol. Therefore, the enhanced transcriptional activity of ER-L372R can be explained in part by increased coactivator binding. Given the overlapping nature of the corepressor and coactivator binding site on ERα, it is likely that the increased transcriptional activity of ER-L372R is due to both a loss of corepressor binding and an increase in the ability of coactivators to bind to the receptor.

- **The ER-L372R mutant is not hypersensitive to estradiol**

The dramatically enhanced estradiol-dependent transcriptional activity of ER-L372R prompted us to examine whether this mutant receptor displayed an increased sensitivity to estradiol. To address this issue, HepG2 cells were transfected with 10 ng of either the wild-type ERα or ER-L372R expression vector along with 3xERE-TATA-Luc reporter and subsequently treated with increasing concentrations of estradiol (Figure 3). Contrary to what was expected, we observed that the mutant receptor was about one order of magnitude less sensitive to estradiol than the wild-type receptor. These results suggest that mutation at Leu-372 of ERα did not render the receptor more sensitive to estradiol treatment.

- **ER-interacting CoRNR peptides show distinct preferences for other nuclear receptors**

It has been shown that different members of the nuclear receptor superfamily display distinct preferences for the known IDs (receptor-interacting domains) located within NCoR and SMRT (6). Consequently, we examined the binding specificity of the ER-interacting CoRNR peptides with other nuclear receptors using the mammalian two-hybrid assay. Surprisingly, although these peptides were designed using the CoRNR box consensus derived from NCoR and SMRT, which interacts with both apo-RARα and apo-TRß, none of the CoRNR box-containing peptides obtained in this study interacted with these two receptors. However, we noticed that the bN2 peptide can interact with apo-RXRα, and treatment with 9-*cis*-retinoic acid abolished this interaction (Figure 4). Under the same conditions apo-RXR showed only minimal binding to NCoR/SMRT IDs, and the interaction between SMRT ID-C and apo-RXR was actually slightly

increased, rather than inhibited, by retinoic acid treatment, consistent with previous observations that RXRα only weakly interacts with SMRT, and retinoic acid treatment can strengthen this interaction (6, 7). The binding of progesterone receptor (PR) to ER-interacting CoRNR peptides was also examined. Figure 4 showed that no interaction of PRA and PRB with the CoRNR peptides was observed in the absence of hormone or in the presence of progesterone. However, treatment with RU486 (a mixed agonist/antagonist) or ZK98299 (a pure antagonist) facilitated a robust interaction between both PR isoforms and the bT1 peptide. As antagonist-bound PRs can interact with NCoR/SMRT IDs, as shown in Figure 4, and in previous studies (8), it is not surprising that CoRNR box-containing peptides with this binding specificity were found in our screens. However, there are differences in the CoRNR box binding patterns of PR treated with RU486 or ZK98299. The bT1 peptide, which binds tamoxifen-bound ERα and ERß, associates more strongly with PRs treated with RU486 than ZK98299, whereas NCoR/SMRT ID-Cs exhibit a stronger interaction with ZK98299-bound PRs than RU486-bound PRs. These results demonstrated that the flanking sequences of the CoRNR box can dictate the receptor binding specificity and that an unidentified corepressor(s) other than NCoR/SMRT might exist that shares the CoRNR box motif, but differentially interacts with different nuclear receptors.

Key Research Accomplishments

May 2002 – April 2001:

- Used phage display screening to identify peptides that bind apo-ER or antiestrogen-ER with high specificity and affinity using CoRNR box focused library.
- Confirmed the interaction of CoRNR box-containing peptides identified in the phage display screens with ER in mammalian cells.
- Defined the region on ER required for CoRNR box-containing peptide binding.

May 2003 – April 2004:

- Used the CoRNR peptides identified in the phage display screens to dissect the mechanism of ER actions
- Discovered that ER-specific CoRNR peptides have different characteristics from those of the CoRNR peptides derived from corepressors NCoR and SMRT
- Generated a contruct for expression of human RARα in insect cells
- Expressed and purified human RARα for phage display screens
- Identified apo-RAR-specific peptides that exhibit same binding characteristics similar to those of the CoRNR box-containing peptides derived from corepressors NCoR and SMRT
- Showed that overexpression of the RAR-specific CoRNR peptides can increase the basal transcription rate of a RARE-TK-Luc construct.

May 2004 – April 2005:

- Demonstrated that the increased interaction with known LXXLL motif-containing peptides may partly account for the enhanced transcriptional activity of the ER-L372R mutant
- Demonstrated that the ER-L372R mutant is not hypersensitive to estradiol
- Discovered that ER-interacting CoRNR peptides show distinct preferences for other nuclear receptors

Reportable Outcomes

Manuscripts:

- Chang C.-Y., Norris J.D., Jansen M., Huang H.-J., McDonnell D.P. (2003) Application of random peptide phage display to the study of nuclear hormone receptors. *Methods in Enzymology* 364: 118-142.
- Huang H.-J., Norris J.D., McDonnell D.P. (2002) Identification of a negative regulatory surface within estrogen receptor alpha provides evidence in support of a role for corepressors in regulating cellular responses to agonists and antagonists. *Molecular Endocrinology*, 16: 1778-1792.

Meeting Abstracts:

- Huang H.-J., McDonnell D.P. (2003) Development of peptide antagonists that target retinoic acid receptor alpha-corepressor interactions. Poster presentation at the 85th Annual Meeting of the Endocrine Society, Philadelphia, PA.
- Huang H.-J., Norris J.D., McDonnell D.P. (2002) Identification of a negative regulatory surface within estrogen receptor alpha provides evidence in support of a role for corepressors in regulating cellular responses to agonists and antagonists. Poster presentation at Keystone Symposia on Nuclear Receptor Superfamily, Snowbird, UT.

Scholarship:

- Keystone Symposia scholarship, 2002

Conclusion

The main goal of this proposal is to use combinatorial phage display to screen for corepressor-like peptides (CoRNR box-containing peptides) that bind selectively to apo-ER, tamoxifen-bound ER, and ICI 182,780-bound ER and to use these peptides to further understand the molecular mechanism of ER action. We have successfully completed Task 1 and identified three different classes of CoRNR peptides whose ability to interact with ER was influenced by the nature of the bound ligand. As shown in the previous two annual reports and in this final report, these CoRNR peptides have helped map a negative regulatory surface within ERα. Our findings are consistent with the existence of corepressors that interact with and modulate ERα activity. These studies have provided a better understanding of ER action and the assays developed from this research will be valuable for the design of screens for novel ER-antagonists for the treatment of breast cancer.

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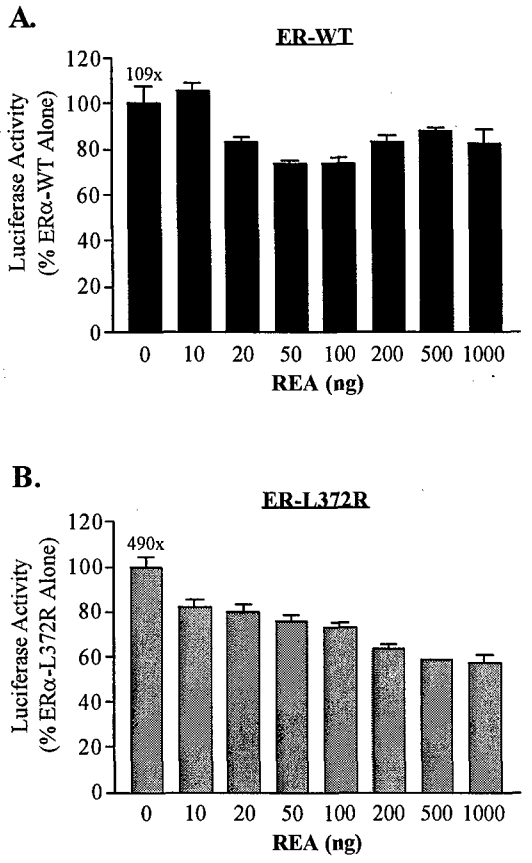

Figure 1. REA suppresses the transcriptional activity of wild-type ER and ER-L372R. HepG2 cells were transfected with 10ng of (A) wild-type or (B) mutant ERα along with 3xERE-TATA-Luc reporter, the β-galactosidase control plasmid, and an increasing concentrations of REA. Following transfection, cells were treated with estradiol (100 nM) for 24 h before harvested for luciferase and β-galactosidase activity. Luciferase activity is presented relative to the response estradiol in the presence of the ER alone, which is set at 100%. Numbers at the top of the leftmost bar show the fold induction in luciferase activity by estradiol in the absence of added REA.

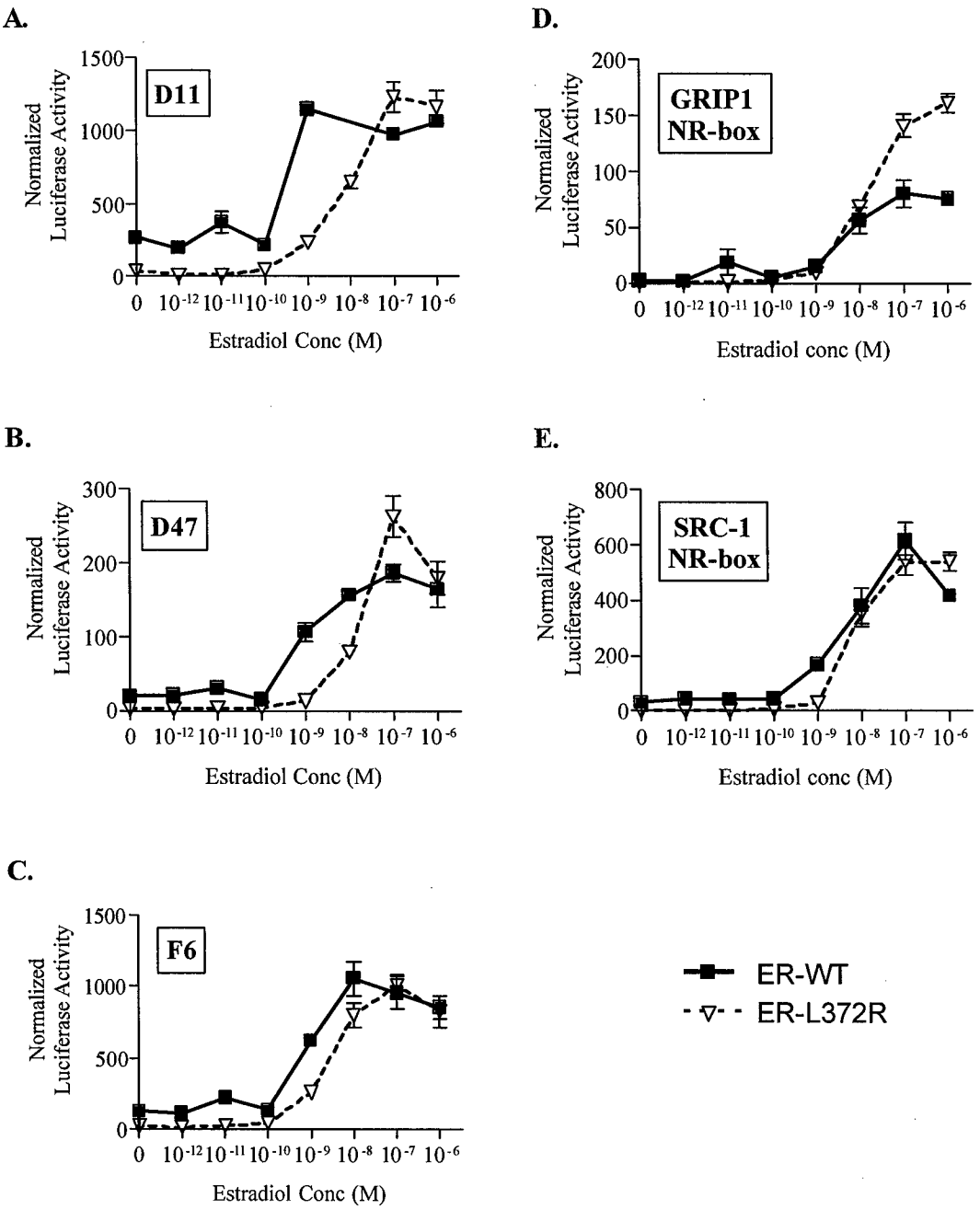

Figure 2. ER-L372R Shows Higher Capacity for Binding to LXXLL Motif-Containing Peptides. VP16-ER-WT or VP16-ER-L372R was transfected into HepG2 cells along with 3xERE-TATA-Luc, the ß-galactosidase control plasmid, and Gal4-DBD expressing different LXXLL motif-containing peptides as indicated in each panel. After transfection, cells were treated with increasing concentrations of estradiol for 24 h before being harvested for determination of luciferase and ß-galactosidase activities. Data are presented as normalized luciferase activity, which was obtained by dividing luciferase activity by ß-galactosidase activity.

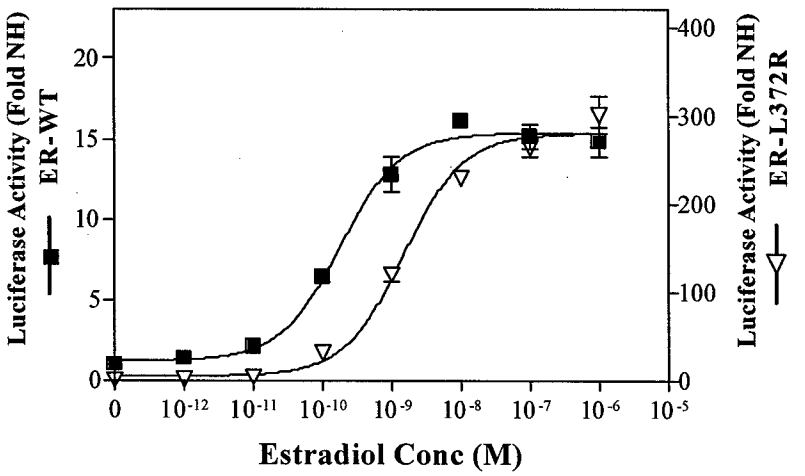

Figure 3. Dose-Response Analysis of 3xERE-TATA-Luc Induction by Estradiol for the wtER or Mutant ER-L372R. HepG2 cells were transfected with 10 ng ER-WT or ER-L372R along with 3xERE-TATA-Luc and the ß-galactosidase control plasmid. After transfection, cells were treated with increasing concentrations of estradiol for 24 h before being harvested for determinations of luciferase and ß-galactosidase activities. Data are presented as fold induction, which was obtained by dividing the normalized luciferase activity in the presence of ligand by that in the absence of ligand. Note that the scale for ER-WT (*left* y-axis) is about 1/20th that for ER-L372R (*right* y-axis).

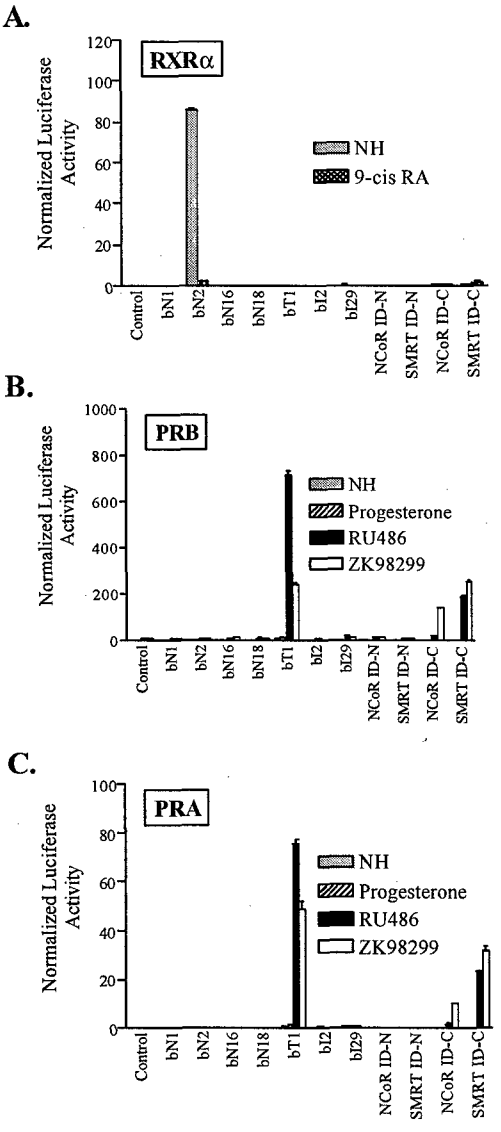

Figure 4. ER-Interacting CoRNR Peptides Show Distinct Preferences for Other Nuclear Receptors. The ability of the CoRNR box-containing peptides to interact with RXRα (A), PRB (B), or PRA (C) was tested using mammalian two-hybrid assays. HepG2 cells were transfected with VP16-receptor fusion expression vector along with the Gal4-DBD-peptide fusion construct, the 5xGal4-TATA-Luc reporter, and the ß-galactosidase control plasmid. After transfection, cells were treated with 100 nM hormone for 24 h before being harvested for determination of luciferase activity. Data are presented as normalized luciferase activity, which was obtained by dividing luciferase activity by ß-galactosidase activity.